Original Research Article

ASSOCIATION OF ENDOTHELIAL NITRIC OXIDE SYNTHASE GENE POLYMORPHISMS T-786C & 27bp (4b/4a) WITH OBESITY IN EGYPT

Abstract:

Background and Objective: Endothelial nitric oxide synthase gene polymorphism (eNOS) is one of three isoforms that synthesize nitric oxide (NO), that participates in several biological processes have been associated with obesity. This study was undertaken to determine if eNOS gene (T786C) and 27bp (4b/4a) were associated with susceptibility of obesity. **Materials and Methods:** The study was carried out on 200 cases divided into 100 obese patient and 100 healthy as control. The mean age cases was (27.02 ± 10.90) they include 79 female and 21 males. All participants were subjected to an estimation of their body mass index (BMI), weight hip ratio (WHR), in addition to random blood sugar (RBS), total cholesterol, triglyceride (TG), and lactate dehydrogenase enzyme (LDH). DNA was amplified using PCR-SSP for detection of relation between polymorphism and endothelial nitric oxide synthase gene in two parts T786C and 27bp (4b/4a).**Results:** All cases showed that there were significant difference between cases and controls regarding to their chemical lab's analysis (TG, Cholesterol, LDL and HDL).All cases showed significant frequency of T786C TT, CC, TC vs. controls ($p<0.001$) these was considered risk factor for disease. On the other hand there no significant difference between 27bp aa, bb, and ab ($p=0.618$) vs. controls. **Conclusion:** The polymorphism T786C not the 27bp in eNOS was associated with obesity.

Key words: Endothelial Nitric oxide, gene, polymorphism, obesity.

Abbreviations: *Endothelial nitric oxide (eNOS), polymerase chain reaction with sequence specific primers PCR-SSP. Nitric oxide NO.*

Introduction

Obesity is a medical condition in which surplus body fat accumulated to the range that it might had a negative effect on health **(1),** people are generally considered obese when their body mass index (BMI), a measurement obtained by dividing a person's weight by the square of the person's height, is over 30 kg/m^2, with the range 25–30 kg/m^2 defined as overweight **(1)**, some East Asian countries use lower values **(2),** obesity increases the incidence of various diseases and conditions, specially cardiovascular diseases, type 2 diabetes, obstructive sleep apnea, definite types of cancer, osteoarthritis and depression **(3), (4).**

Obesity is most commonly caused by a mixing of excessive food intake, lack of physical activity, and genetic susceptibility **(1),(5),** a few cases are caused firstly by genes, endocrine disorders, medications, or mental disorder **(6),** on the other hand obese people eat little next to gain weight because of a slow metabolism is not medically supported***(7)***, on average, obese people have a greater energy usage than their normal people because of the energy required to maintain an increased body mass **(7),(8).**

Obesity might be a cause of death which can be preventable worldwide, with increasing rates in adults and children ***(1).*** In 2015, 600 million adults (12%) and 100 million children were obese in 195 countries. ***(9)*** Obesity is more common in women than men ***(1)***. Several studies viewed that obesity is one of the most dangerous public health problems of the 21st century. ***(10)*** In 2013, obesity is classified as a disease by the American Medical Association***. (11), (12).***

Impaired nitric oxide production is involved in the pathogenesis of several diseases such as hypertension, diabetes mellitus, obesity, erectile dysfunction, and migraine **(13),** a large number of studies showed that polymorphisms in NOS_3 gene affect the susceptibility to these diseases **(13),** although NOS_3 is a highly polymorphic gene, three genetic polymorphisms in this gene have been widely studied: the single nucleotide polymorphisms (SNPs) g.-786T>C located in NOS_3 promoter and in exon 7, respectively, and the variable number of tandem repeats 4b/4a (VNTR) characterized by 27 bp repeat in intron 4 ***(13).*** The C allele for the T786C polymorphism, which results in reduced eNOS expression and nitric oxide production was associated with increased risk for hypertension ***(14).*** The VNTR in intron 4 affects eNOS expression ***(15).*** And the susceptibility to hypertension ***(14).*** Obesity ***(16).***

Materials and Methods

Study group: This study includes 200 cases 100 obese patients they were recruited from the Department of Diabetes and Endocrine Unit in Specialized Medical Hospital Mansoura University, Egypt as well as Ministry of Health Hospitals of Dakahlia, Egypt during the period September 2016 to May 2018, and 100 healthy control. The mean age of cases were 27.02 ± 10.90 years they were in the form of 21 male and 79 female. According to the definition of metabolic syndrome given by WHO, ATP and IDF (75%) of patient were classified as having metabolic syndrome while the rest, (25%) were not complicated and were characterized as just having simple obesity.

Control group: For comparison 100 healthy controls were selected.

Biochemical analysis: After 12 h of fasting, a blood was collected from each case and control in an empty tube blood sample for biochemical analysis. If the sample were not analyzed immediately, they will frozen and stored at -70 C. Total cholesterol, triglyceride (TG), LDL and HDL were measured by enzymatic methods on automatic biochemistry analyzer.

Capture column kit extraction and purification:

The generation DNA purification capture column kit (Gentra System, USA) is based on a proprietary system that uses two reagents, a DNA purification solution and a DNA elution solution, along with a specially formulated purification matrix. In this kit , a sample is applied directly to the purification matrix contained a spin column .the cells contained in sample lyse upon contact with the matrix .once the cells were lysed, DNA was captured by the matrix material which make it possible to efficiently wash away contaminants ,leaving the DNA bound to the matrix. Contaminants, including protein heme and RNA were removed from the matrix by washing with DNA purification solution.

Following removal contaminants, the DNA released from the matrix using DNA elution solution and heat .Samples of purified DNA were ready for analysis and not require precipitation.

PCR amplifications of each eNOS studied: Single nucleotide polymorphism (SNPs) for nitric oxide synthase gene (eNOS) were genotyped in this case-control study C786Tand 27bp polymorphism using polymerase chain reaction PCR. Amplification were performed in sequence-specific primer polymerase chain reaction (SSP-PCR) employing a forward and reverse primer for each part. The region containing one (Restriction Fragment Length Polymorphisms) RFLPs within the eNOS gene was amplified with tag DNA polymerase, PCR buffer, Mgcl2 and dNTPs.

The entire reaction volume plus 5 μL of bromophenol blue track dye were loaded into 2% agarose gel (Bohringer Mannheim) containing ethidium bromide. And for 30 minutes at 100V Gels were electrophoresed, then photographed under UV light (320 nm) and then detect the presence or absence of an allele specific bands.

Primer sequences and PCR condition of eNOS gene polymorphism:

The T786C genotype was performed using PCR amplification, the amplified product was digested with NgoMIV enzyme. Briefly primer sequences were forward primer: 5'-ATG CTC CCA CCA GGG CAT CA-3' and reverse primer: 5'-GTC CTT GAG TCT GAC ATT AGG G-3'.

The 27 bp (4b/4a) was determined using PCR amplification, not followed by restriction enzyme digestion of the amplified product. Briefly primer sequences were forward primer: 5'AGG CCC TAT GGT AGT GGC CTT T-3' and reverse primer: 5'TGC TCC TGC TAC TGA CAG CA-3'

Statistical analysis:

Statistical analysis of data was done using the software statistical package (SPSS program version 17). The student t-test was used to compare the numerical values related to cholesterol, other chemical parameter and body

mass index whereas CHI square test used to compare frequencies of different genotypes and alleles between cases and controls.

Results

Cases and controls showed a non-significant difference regarding to their age (p = 0.74). However, cases showed a significant levels of BMI, cholesterol, TG, HDL-C and LDL-C (p <0.001). (Table 1)

Regarding to descriptive data of studied cases of obesity, cases showed a significant difference vs. control (normal, no disease) with p <0.001 (Table 2)

Regarding to distribution of eNOS gene polymorphism (T786C) (table 3): all genotypes (TT), (TC), and (CC) were highly significant (p<0.001) vs. controls. While on alleles analysis both (T) and (C) were significantly. (p <0.001)

Comparing all cases with obesity and healthy controls regarding their genotype distribution of eNOS gene polymorphism (27 bp), (table 4): all genotypes (aa), (ab), and (bb) were non-significantly (p=0.618) vs. controls. While on alleles analysis (a) and (b) did not show any significant difference. (p =0.482).

Table 1: Descriptive data of studied cases of obesity and healthy controls.

	Patients (N=100)	**Control (N=100)**	**t**	***P***
Hip	122.69 ± 12.96	89.26 ± 17.18	15.536	<0.001*
Weight	106.03 ± 16.95	68.66 ± 17.77	15.216	<0.001*
Height	162.47 ± 8.26	166.38 ± 7.55	3.495	0.001*
BMI	40.13 ± 6.40	25.02 ± 7.67	15.132	<0.001*
WHR	0.95 ± 0.14	0.82 ± 0.12	7.268	<0.001*
waist	116.16 ± 15.47	74.57 ± 24.76	14.245	<0.001*
Age	27.02 ± 10.90	27.51 ± 10.26	0.327	0.744
Cholesterol	246.32 ± 60.23	181.16 ± 44.48	8.703	<0.001*

TG	140.76 ± 95.91	101.74 ± 47.85	3.640	<0.001*
HDL-C	49.94 ± 15.60	37.54 ± 13.48	6.014	<0.001*
LDL-C	168.85 ± 64.86	124.10 ± 40.89	5.835	<0.001*

N: number of cases, t: Student t-test, TG: Triglyceride, HDL: High-density lipoprotein, LDL: Low-density lipoprotein, *p =0.001 (significant).

Table 2: descriptive data of studied cases of obesity.

	Patients		**Control**		**χ^2**	***p***
	N	**%**	**N**	**%**		
disease						
obesity	53	53.0%	0	0.0%	200.000	<0.001*
obesity+D.M	21	21.0%	0	0.0%		
obesity+HTN	12	12.0%	0	0.0%		
obesity+D.M+ HTN	14	14.0%	0	0.0%		
normal, no disease	0	0.0%	100	100.0%		

N: Number of cases, %: percentage of cases, χ^2: Chi-square test

D.M.: Diabetes Mellitus, HTN. : Hypertension

Tablet 3: comparison between all cases with obesity and healthy controls regarding their genotype distribution of eNOS gene polymorphism in (T786C).

T786C		**Patients**		**Control**		**χ^2**	***p***
		N	**%**	**N**	**%**		
Genotype	TT	33	33.0%	84	84.0%	53.736	<0.001*
	TC	55	55.0%	14	14.0%		
	CC	12	12.0%	2	2.0%		
Alleles	(T)	121	60.5%	182	91%	50.641	<0.001*
	(C)	79	39.5%	18	9%		

N= number of cases, % = percentage of cases, TT = thymine thymine, TC =thymine cytosine, CC= cytosine cytosine, T =thymine, C=cytosine. Significance using $\chi 2$: Chi-square test:

*p<0.001 (significant)

Tablet 4: comparison between all cases with obesity and healthy controls regarding their genotype distribution of eNOS gene polymorphism in (27 bp) repetition

27bp		Patients		Control		$\chi 2$	*p*
		N	**%**	**N**	**%**		
Genotype	aa	15	15.0%	14	14.0%	0.961	0.618
	ab	63	63.0%	58	58.0%		
	bb	22	22.0%	28	28.0%		
Alleles	(a)	93	46.5%	86	43%	0.495	0.482
	(b)	107	53.5%	114	57%		

N= number of cases, % = percentage of cases, a=allele a, b= allele b

Significance using $\chi 2$: Chi-square test.

Electrophoresis result of PCR showing enzymatic digestion of T786C polymorphism of eNOS gene:

Wild type TT is found which appear at 236bp in lanes 1, 2, 4 and 5, digestion of PCR product of T786C polymorphism of eNOS gene using NgoMIV enzyme. Which digest the 236-bp fragment into 203 and 33-bp fragments (heterozygous mutated genotype TC which has 236, 203, 33 bp fragments lanes 6 only) but (homozygous mutated genotype CC is found which has 203, 33 bp fragments lanes 3, 7) by using DNA size marker 50bp

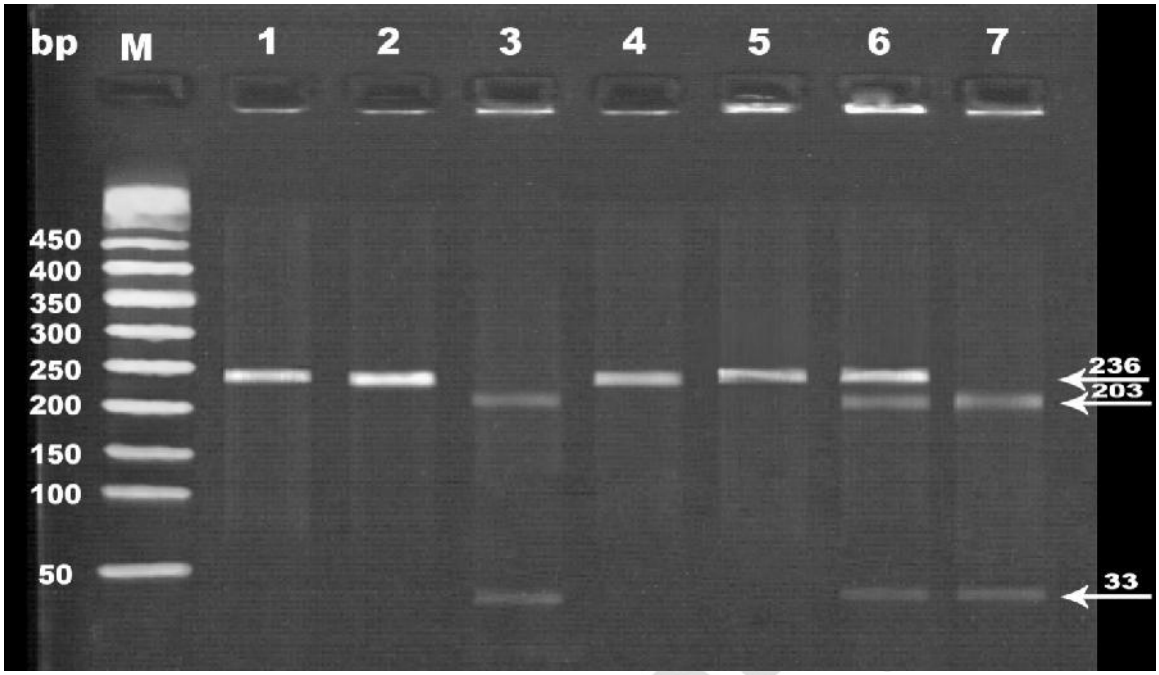

Fig 1: Enzymatic digestion of T786C polymorphism of eNOS gene.

Electrophoresis result of PCR showing PCR amplification of Intron 4b/a (27bp) polymorphism of eNOS gene:

PCR product of intron 4b/a polymorphism have ban size (220) bp in bb carrier homozygous lanes 2, 3, 6 and 7 and have ban size (193) in aa homozygous lanes 4 and ba carrier heterozygous which has (220,193 bp fragments lanes 1 and 5) by using DNA marker 50 bp.

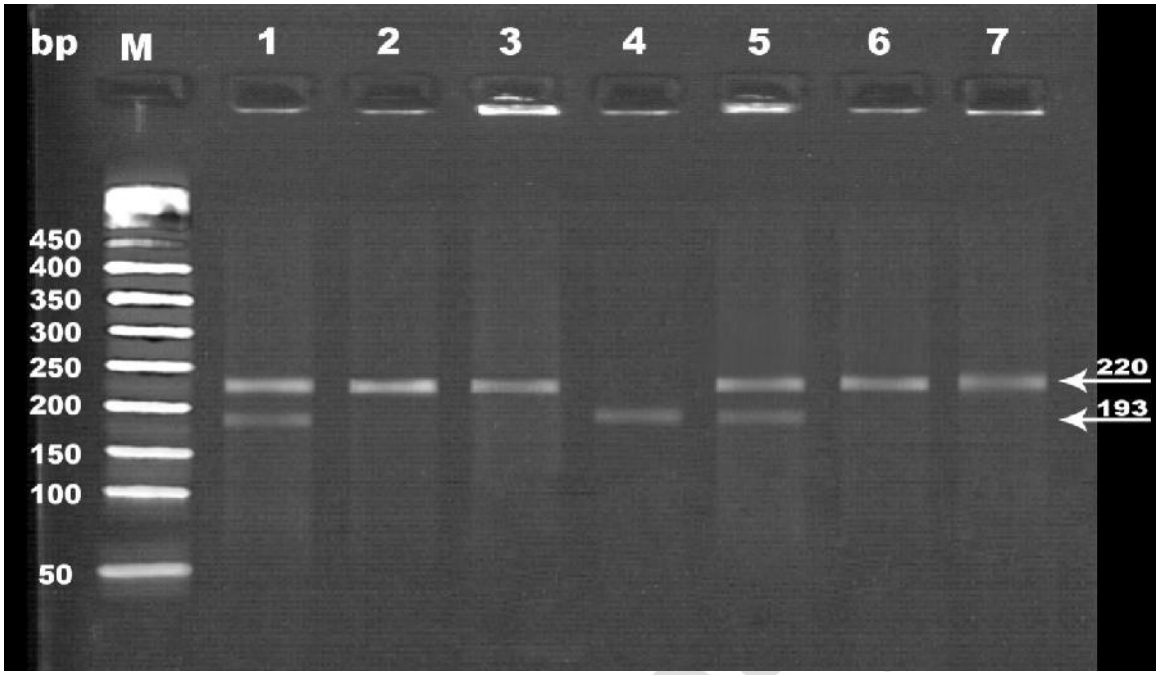

Fig 2: PCR amplification of intron 4b/a polymorphism of eNOS gene

Discussion

Overweight and obesity are major risk factors for a number of chronic diseases, including diabetes, cardiovascular diseases and cancer. Once considered a problem only in high income countries, overweight and obesity are now dramatically on the rise in low- and middle-income countries **(1).**

Obesity is one of the leading preventable causes of death worldwide ***(17), (18).***Growing evidence supports the association of diseases with NOS_3 haplotypes (combination of alleles in close proximity, within a DNA block). This approach may be more informative than the analysis of genetic polymorphisms one by one ***(19).*** Haplotypes including the SNPs g.-786T>C and Glu298Asp, g- G894T and the VNTR in intron 4 affected the susceptibility to hypertension **(20).** And there is association between NOS_3 and the susceptibility to obesity **(16).** And diabetes mellitus **(21).**

The present study aims mainly to investigate the association of the eNOS gene polymorphism (T786 C, and 27bp) with the possibility of occurrence obesity, the study results showed that homozygous mutated TT and homozygous mutated CC genotypes, mutant T and C allele of T786C polymorphism had significant frequency among cases of obesity compared with controls. On the contrary, homozygous mutated bb and homozygous mutated aa genotypes, mutant b and a allele of 27 bp polymorphism had no significant frequency among cases of obesity compared with controls.

Souza-Costa DC.et al. (16) A Brazilian study suggested that the eNOS gene polymorphism is associated with hypertension in obese children and adolescents. Further studies examining the possible interactions of eNOS haplotypes with environmental factors and other genetic markers might cause the development of obesity and its complications are warranted.

The present research exhibited a significant association of T786C with occurrence of obesity and these results in harmony with results of **Josiane A. Miranda et al. (22)** reported a similar association of the T786C polymorphism predispose to MetS in both obese children and adolescents.
Bressler J. et al. (23) in the United States in a study carried in four communities suggested that interaction between incidence of obesity and NOS_3.

In partial agreement with our result **Baráth A et al. (24)** have reported that no significant differences were seen in the case of the eNOS 4th intron 27-bp repeat polymorphism and the eNOS T-786C promoter polymorphism.

On other hand to our result, **Roberta Fernanda da Silva et al.(*25)*** a study on Brazilian patients did not demonstrate a significant difference in plasma NO2 concentration blood pressure and obesity taking into account the haplotype results (-786T/C, 4b/4a, and 894G/T). In general, different levels of Training status promote different results in these variables; however, these relationships need to be studied further.
On the contrary to the present research **Hela Ben Nasr et al. (26)** suggested that among Tunisian patients, eNOS gene polymorphism 27pb (4b/a) was significantly associated with obesity.

Our study reported that endothelial nitric oxide gene polymorphism (T786C) is a risk factor for development of obesity

CONCLUSIONS

The C786T polymorphism of eNOS gene was found to be significantly associated with development of obesity .and T, C alleles, (CC and TT genotypes of C786T) might significantly considered genetic risk factor for development of obesity.

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ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been

performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Consent Disclaimer:

As per international standard or university standard, patient's written consent has been collected and preserved by the author(s).

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